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## Infection of Laboratory-Colonized *Anopheles darlingi* Mosquitoes by *Plasmodium vivax*

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**Abstract.** *Anopheles darlingi* Root is the most important malaria vector in the Amazonia region of South America. However, continuous propagation of *An. darlingi* in the laboratory has been elusive, limiting entomological, genetic/genomic, and vector–pathogen interaction studies of this mosquito species. Here, we report the establishment of an *An. darlingi* colony derived from wild-caught mosquitoes obtained in the northeastern Peruvian Amazon region of Iquitos in the Loreto Department. We show that the numbers of eggs, larvae, pupae, and adults continue to rise at least to the F6 generation. Comparison of feeding *Plasmodium vivax* *ex vivo* of F4 and F5 to F1 generation mosquitoes showed the comparable presence of oocysts and sporozoites, with numbers that corresponded to blood-stage asexual parasitemia and gametocytemia, confirming *P. vivax* vectorial capacity in the colonized mosquitoes. These results provide new avenues for research on *An. darlingi* biology and study of *An. darlingi*–*Plasmodium* interactions.

### INTRODUCTION

*Anopheles darlingi* Root is the most important malaria vector in the Amazonia region of South America (reviewed in ref. 1). Numerous observational studies have detailed this mosquito species' role in epidemic and endemic malaria transmission in this region. Previous studies have shown that laboratory-reared F1 generation *An. darlingi* derived from wild-caught mosquitoes are suitable for laboratory-based studies of *Plasmodium*–mosquito interactions by both membrane feeding assays and direct feeds.<sup>2–7</sup> An important limitation of such studies is the availability of *An. darlingi* mosquitoes. Hitherto, there has been an inability to propagate *An. darlingi* continuously in the laboratory, hence requiring human landing catches or alternative sampling methods<sup>8</sup> to obtain sufficient mosquitoes for laboratory-based experimental study. Despite a report from the 1940s indicating that *An. darlingi* could be continuously propagated,<sup>9</sup> establishment of a laboratory colony of *An. darlingi*, coupled with experimental infection by human-infecting malaria parasites such as *P. vivax*, has not been reported.

Malaria transmission in the Peruvian Amazon is seasonal, with a peak from January to June, which is coincident with the rainy season.<sup>10,11</sup> Similarly, anopheline densities are seasonal, typically peaking from March to May in the Iquitos region. Reported human biting rates (HBRs) are as high as 750 bites/night for *An. darlingi*, but a lower HBR (~10) has been detected from August to December (Moreno M and Conn JE, unpublished data). Recent observations indicate that some areas of the Peruvian Amazon have infected biting rates by *An. darlingi* as high as reported from some parts of sub-Saharan Africa.<sup>12</sup> Therefore, mosquito collections during the low malaria season limit various research activities, and the logical approach would be to colonize and maintain *An. darlingi* in the laboratory.

In 1947, in British Guiana, the first effective effort to colonize *An. darlingi* was reported, with success in 35 generations

and natural mating under laboratory conditions.<sup>9</sup> Later, some Brazilian populations of *An. darlingi* were reported to be colonized in the laboratory up to 10 years<sup>13</sup> as well as for a shorter period of time.<sup>14</sup> Both studies underscored the importance of cage size and density of specimens per experiment to obtain fertilized females and avoid the forced mating technique. Some researchers have suspected that the challenge in colonizing some mosquito species that might mate in swarms (eurygamy) is to find the right conditions for successful mating obtained by non-artificial methods and generation by generation, select the population for ability to mate in a restricted space (stenogamy),<sup>15</sup> although capture–recapture evidence has indirectly suggested that *An. darlingi* is not obligately eurygamous.<sup>16</sup> To date, no *An. darlingi* colony strain has been permanently established or is available for research purposes.

Detailed study of *An. darlingi* biology, genomics (using inbred, genetically homogenous mosquitoes),<sup>17</sup> and mechanistic studies of *Plasmodium*–*An. darlingi* interactions, therefore, have been limited by the hitherto inability to establish the continued propagation of this key mosquito species in the laboratory. Here, we describe the establishment of an *An. darlingi* colony and show the experimental infection of these colonized mosquitoes by *P. vivax* through artificial membrane feeding using parasitized blood obtained *ex vivo* from infected humans in the Peruvian Amazon. The importance of this work is furthered by the lack of known transovarially transferred pathogens in this mosquito species; hence, the possible future use of direct mosquito feeding on infected patients<sup>18,19</sup> would be considered ethical and safe.

### MATERIALS AND METHODS

**Obtaining field-caught *An. darlingi*.** In total, 135 adult females were collected in July of 2013 by human landing catch in Cahuide (04°13.785' S/073°276' W), a village located along the Iquitos–Nauta road 60 km from Iquitos city in the Peruvian Amazon. This village is on the banks of the Itaya River, a tributary of the Amazon River, with suitable habitat for *An. darlingi* breeding. Mosquitoes were maintained in cups with 10% sugar solution and transferred to the laboratory;

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then, they were morphologically identified using established entomological keys.<sup>20</sup>

**Mosquito husbandry.** Eggs from each female were placed into independent round oviposition containers (9-cm diameter, 7-cm deep) lined with wet filter paper. Two days after hatching, larvae were transferred into a pan (33 × 22 × 5 cm) containing 200 larvae per tray for larvae stages I and II and 100 larvae per tray for stages III and IV. Larval food was a mixture (by weight) of fishmeal (24%), wheat flour (13%), corn flour (13%), maca powder (*Lepidium meyenii*; 13%), soybean meal (24%), and cornstarch (13%; ~30% protein). Food was provided one time daily for larvae in stages I and II and three to four times daily for larvae in stages III and IV. Pupae were removed daily and placed into plastic containers in a screened cage (46 × 46 × 46 cm) for adult emergence. Adults were given 10% sugar solution and maintained in controlled conditions at 27°C, 80% relative humidity, and a 12/12-hour day/night photoperiod.

**Standard membrane feeding assay.** Subjects presenting with acute symptomatic malaria caused by microscopically determined *P. vivax* infection were the source of parasitized blood for feeding to mosquitoes as previously described.<sup>3</sup> No *P. falciparum* asexual or gametocyte forms were seen by light microscopy. Blood was obtained in citrate and centrifuged; then, plasma was removed and replaced with an equivalent volume of plasma pooled from 10 donors with no history of malaria.

This study was approved by the Human Subjects Protection Program of the University of California at San Diego (La Jolla, California) and the Comité de Ética of the Universidad Peruana Cayetano Heredia (Lima, Peru).

Oocysts were enumerated using light microscopic examination of unstained dissected midguts. Sporozoites were obtained by first removing the mosquito head and then cutting and triturating the region of the upper thorax where the salivary glands are found. This material was pooled and centrifuged, and sporozoites were enumerated using a Petroff-Hausser counting chamber under 400× magnification.

**Induction of natural mating.** Previously published protocols for establishing natural mating of *An. pseudopunctipennis* were adapted to induce natural mating of *An. darlingi*.<sup>21,22</sup> Equal numbers of freshly emerged male and female (total of ~1,000) adults were placed into a cage of 46 × 46 × 46 cm, and the ambient temperature was lowered to 24°C. Either a blue stroboscopic light source (Opaluz strobe warming light [30 W])<sup>22</sup> or an automated 40 lumens white light flashlight (Opalux Flash 40 LED)<sup>21</sup> was used to shine into the mosquito cages to induce mating. Each light treatment was carried out for two cycles of 20 minutes of light on alternated with 10 minutes of light off, which was carried out for 7 consecutive evenings, just after dusk, between 6:30 and 7:00 PM. On days

6 and 7, commercially purchased warmed chicken blood was provided through membrane feeders to female mosquitoes; sugar solution had been withheld for days 4 and 5. After blood fed, mosquitoes were restarted on sugar water 3 days later. On day 9 post-emergence, an additional blood meal was provided. Unfed mosquitoes were discarded. Forty-eight hours after blood feeding, oviposition was induced by cutting one wing (with a 21-gauge needle) of ethyl acetate-anesthetized mosquitoes. Recovering mosquitoes were placed individually into plastic vials containing a humidified, filter paper-covered cotton ball.

## RESULTS

**Establishment of continuous laboratory-based production of *An. darlingi*.** Previous work from Iquitos, Peru has described infecting F1 *An. darlingi* mosquitoes with *P. vivax* obtained *ex vivo* from infected humans in the Peruvian Amazon.<sup>3,4</sup> To start the present colony, F1 generation mosquitoes were obtained by feeding wild-caught mosquitoes purchased fresh chicken blood to induce egg laying (Table 1). Alterations in the light and temperature laboratory conditions as reported previously for *An. pseudopunctipennis*<sup>21</sup> produced stable and increasing numbers of *An. darlingi* eggs, larvae, pupae, and adults (with expected male to female ratios) through six generations as of the time of this writing.

**Comparison of type of light stimulation with outcome of *An. darlingi* mating.** Previous experiments with *An. pseudopunctipennis* in Bolivia and Mexico have used both stroboscopic blue light and pulses of white light from a standard flashlight to attempt to induce this species to mate.<sup>21,22</sup> In two experiments with F2 generation mosquitoes, we compared these two types of light on the efficiency of oviposition, number of eggs laid, and larval hatching. Head to head comparisons showed no difference in the total number of ovipositions ( $N = 159$  and  $N = 110$  for white flashlight;  $N = 121$  and  $N = 231$  for blue stroboscopic light). The proportion of ovipositions with the two conditions was similar: 20% and 26%, respectively, with the white flashlight compared with 21% and 22%, respectively, with the blue stroboscopic light. The egg and larval yields did not differ between the two conditions.

**Experimental infection of colonized *An. darlingi* with *P. vivax* obtained *ex vivo* from humans.** Because it is possible that laboratory adaptation of *An. darlingi* might lead to a founder effect-related line of mosquitoes refractory to *P. vivax* infection—at the level of either oocysts or sporozoites—we compared experimental infection of F1 with F4, F5, and F6 generations of mosquitoes (Table 2). The key observation is that both oocysts and sporozoites developed as well in all three of these generations as in F1 mosquitoes. The number

TABLE 1  
Laboratory-based *An. darlingi* oviposition and hatching efficiency by generation

Generation	Total number of mosquitoes	Total number of oviposition (%)	Number of eggs laid	Number of hatched larvae (%)	Number of pupae (%)	Number of adults* (%) emerged
F2†	126	22 (17)	1,972	1,542 (78)	1,307 (85)	557/484 (80)
F3	195	32 (16)	2,416	1,756 (73)	1,585 (90)	792/722 (96)
F4	111	49 (44)	4,192	3,027 (72)	2,680 (89)	1,275/1,161 (91)
F5	185	51 (28)	3,203	2,258 (71)	1,718 (76)	789/761 (90)
F6	196	42 (21)	2,564	1,869 (73)	1,719 (92)	1,548 (90)

\* Male/female adults.

† Generation F1 arose from wild-caught *An. darlingi* after blood feeding (chicken blood). Generation F2 was the first entirely laboratory-based generation.

TABLE 2  
Infection of colonized *An. darlingi* by *P. vivax*

Infection number	Generation number	Date of infection (month/year)	Donor gametocytemia	Donor asexual parasitemia number ( $\mu$ L)	Number of midguts dissected/with oocysts (%)	Oocyst number (geometric mean)	Number of sporozoite/mosquito*
1	F1	9/2013	3,606	3,060	25/36 (69)	57	Not done†
	F3				27/31 (87)	54	Not done†
2	F1	9/2013	2,078	14,810	22/35 (63)	34	933
	F3				25/34 (74)	39	1,200
3	F1	10/2013	118	186	13/38 (34)	1.4	170
	F4				12/36 (33)	1.1	150
4	F1	10/2013	330	2,280	11/20 (55)	1.2	720
	F5				9/20 (45)	1.1	540
5	F1	11/2013	300	2,910	17/18 (94)	14.1	1,436
	F5				12/14 (86)	10.8	1,260
6	F1	11/2013	360	3,750	15/15 (100)	25.2	7,380
	F6				16/16 (100)	28.5	6,300

\*Sporozoite counts averaged from pooled mosquitoes.

†Only examined for oocysts.

of oocysts and sporozoites was associated with parasitemia levels of the donor patients (Table 2).

## DISCUSSION

This study adapted two protocols for the successful colonization of *An. darlingi* by natural mating under laboratory conditions, although previously, the stroboscopic light approach was unsuccessfully tested with *An. darlingi* from Bolivia.<sup>21,22</sup>

Among the different issues encountered in the establishment of an anopheline colony, mating is the most problematic.<sup>23</sup> In our case, forced mating techniques with this species were unsuccessful, with visual forced mating confirmed but spermathecae found to be negative for insemination when visualized under dark-field microscope (data not shown). Environmental modification to simulate field conditions, such as light changes to simulate natural lighting, temperature, and humidity, have been described.<sup>15,24</sup> However, the evolution of stenogamy of the Cahuide *An. darlingi*-colonized population needs to be addressed for maintenance and the goal of obtaining a self-free mating colony. Laboratory adaptation of *An. darlingi* will presumably lead to changes in genetic composition because of selecting progenies, leading to a homogeneous population structure affected by inbreeding, such as reported for *An. gambiae*.<sup>25,26</sup> Therefore, future analysis will be focus on signatures of population bottlenecks, potential founder effects, and genetic drift in the colony. In addition, different lineages within *An. darlingi* have been detected by microsatellites and nuclear markers across its geographic range.<sup>27,28</sup> Thus, an essential question about differences in transmission arises: is the genetic variation of the mosquito associated with vector refractoriness, or are there lineages more susceptible to *Plasmodium*, for example, such as detected in the neotropical malaria vector complex *An. albitarsis* (reviewed in ref. 29)?

In malaria-endemic regions of the Amazon in South America, the limiting factor for carrying out laboratory-based studies of *An. darlingi* is mosquito availability. Experimental limitations to studying the biology of *P. vivax*-*Anopheles* interactions include access to the non-cultivable gametocytes of *P. vivax* in the same place and time with competent vector mosquitoes.

Although several studies are focused on the refractoriness of different anophelines (*Cellia* or *Anopheles* subgenus) to *P. falciparum* or rodent malaria species, there are very few

reports of the neotropical subgenus *Nyssorhynchus*, particularly *An. darlingi* and *P. vivax* transmission. One of the main unsolved questions in malaria epidemiology in the Amazon Basin is the role of the *P. vivax* asymptomatic parasite carriers in the transmission of disease. An experiment carried out in the Brazilian Amazon showed a 1.2% infection rate of *An. darlingi* from asymptomatic carriers compared with 22% from symptomatic carriers.<sup>30</sup> A study in the Peruvian Amazon revealed differences in mosquito infection depending on gametocytemia from *P. vivax*-parasitemic patients using F1 mosquitoes obtained from an outbred *An. darlingi* population.<sup>3</sup> Here, we report that *An. darlingi* mosquitoes obtained from a colony after five generations were successfully infected with *P. vivax* by artificial membrane feeding. Additional experiments with subsequent mosquito generations will continue to be performed to ascertain the progress of *Plasmodium* susceptibility of this colony. The appearance of refractory mosquitoes would provide the opportunity to carry out crosses to identify potential refractoriness genes in this species.

The recent publication of the *An. darlingi* genome<sup>17</sup> coupled with the availability of a colony could help to study critical aspects related to malaria transmission, such as behavior,<sup>1</sup> host-parasite coevolution,<sup>3,4</sup> susceptibility to *Plasmodium* parasites,<sup>2</sup> testing new drugs against different parasite stages in the mosquito,<sup>31</sup> or genetic determinants of insecticide resistance.<sup>32</sup>

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